A critical role for the nucleus reuniens in long-term, but not short-term associative recognition memory formation

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ABSTRACT

Recognition memory for single items requires the perirhinal cortex (PRH), while recognition of an item and its associated location, requires a functional interaction between the PRH, hippocampus (HPC) and medial prefrontal cortex (mPFC). While the precise mechanisms through which these interactions are effected are unknown, the nucleus reuniens (NRe) has bidirectional connections with each regions and hence may play a role in recognition memory. Here we investigated, in male rats, whether specific manipulations of NRe function affected performance of recognition memory for single items, object location or object-in-place associations. Permanent lesions in the NRe significantly impaired long-term but not short-term object-in-place associative recognition memory, while single item recognition memory and object location memory were unaffected. Temporary inactivation of the NRe during distinct phases of the object-in-place task, revealed its importance in both the encoding and retrieval stages of long-term associative recognition memory. Infusions of specific receptor antagonists showed that encoding was dependent on muscarinic and nicotinic cholinergic neurotransmission, in contrast, NMDA receptor neurotransmission was not required. Finally, we found that long-term object-in-place memory required protein synthesis within the NRe. These data reveal a specific role for the NRe in long-term associative recognition memory through its interactions with the HPC and mPFC, but not the PRH. The delay-dependent involvement of the NRe, suggests that it is not a simple relay station between brain regions, but rather during high mnemonic demand, facilitates interactions between the mPFC and HPC a process which requires both cholinergic neurotransmission and protein synthesis.

Significance statement

Recognising an object and its associated location, fundamental to our everyday memory requires specific hippocampal-cortical interactions, potentially facilitated by the nucleus reuniens (NRe) of the thalamus, yet the role of the NRe itself in associative recognition memory is unknown. Here we reveal the crucial role of NRe in encoding and retrieval of long-term object-in-place memory, but not for remembrance of an individual object or individual location and such involvement is cholinergic receptor and protein synthesis dependent. This is the first demonstration that the NRe is a key node within an associative recognition memory network and is not just a simple relay for information within the network. Rather, we argue, the NRe actively modulates information processing during long-term associative memory formation.

INTRODUCTION

Successful recognition memory depends upon networks of distributed brain regions, with the nature of to-be-remembered information determining which brain regions are recruited. Thus recognition of single items or objects depends on the perirhinal cortex (PRH) (Diana et al., 2007; Kim, 2011; 2013; Wan et al., 1999; Albasser et al., 2010; Ennaceur et al., 1996; Bussey et al., 1999), while recognition of an object and its associated spatial information (object-in-place memory) requires the PRH (Bachevalier and Nemanic, 2008; Bussey et al., 2001; Barker et al., 2007), hippocampus (HPC) and prefrontal cortex (PFC) (Browning et al., 2005; Kesner and Ragozzino, 2003; Brincat and Miller, 2015; Barker et al., 2007; Barker and Warburton, 2008; Barker et al., 2017). Importantly during object-in-place memory these

three brain regions appear to operate cooperatively forming an associative recognition memory network (Barker et al., 2007, Barker and Warburton, 2011). However, as these regions are connected by both direct and indirect anatomical pathways the precise routes by which these neural interactions are effected are largely unknown. One brain region, the nucleus reuniens of the thalamus (NRe) has reciprocal connections with the PRH (Agster et al., 2016; Pereira et al., 2016) mPFC (Vertes, 2002; Vertes et al. 2006; Hoover and Vertes 2006) and HPC (Herkenham, 1978; Wouterlood et al. 1990; Dolleman-van der Weel and Witter, 1996; Vertes et al. 2006). In addition, a proportion of NRe neurons project to both mPFC and HPC, thus NRe may simultaneously influence both HPC and mPFC processing (Hoover and Vertes, 2012) as well as provide a route through which these three brain regions may interact.

There is evidence that the NRe plays a specific role in memory processing under certain conditions. Permanent or temporary inactivation of the ventral midline thalamic nuclei (i.e. NRe and neighbouring rhomboid nucleus (Rh)) impaired a spatial win-shift task (Hembrook and Mair, 2011), a spatial strategy shifting task (Cholivin et al., 2013) and a delayed non-matching to position (Hembrook et al., 2012) task, although such lesions had no effect on simple spatial learning (but see Dolleman-van der Weel et al., 2009). NRe disruption was also found to impair long-term, but not short-term spatial memory (Loureiro et al., 2012). Thus, the NRe plays a key role in spatial memory processing either when the demands on the HPC are high, or interactions between the HPC and mPFC are required. Whether the NRe participates in PRH dependent tasks has not, to our knowledge, been investigated.

In the present study, we hypothesised that the NRe will have a key role in recognition memory formation, either through interactions with the PRH, mPFC, HPC. To test this hypothesis we examined the effects of specific manipulations of the NRe on a series of object recognition

memory tasks. We examined: 1. the necessity of the NRe for single item and associative recognition memory using permanent lesions placed in the midline thalamus; 2. the necessity of the NRe separately during encoding and retrieval; 3. the role of NMDA receptor and cholinergic neurotransmission in the NRe for object-in-place encoding; 4. whether long-term memory maintenance was dependent on protein synthesis in the NRe.

METHODS

Subjects

Experiment 1 used 20 male rats and Experiments 2-5 used a single cohort of 12 male rats, (Lister Hooded strain; Harlan, UK) weighing 300-350g at the start of the experiments. All animals were housed in groups of four, under a 12 h light/dark cycle (light phase, 6.00 P.M to 6.00 A.M) with *ad libitum* access to food and water. Behavioural testing was conducted during the dark phase of this cycle. All animal procedures were performed in accordance with United Kingdom Animals Scientific Procedures Act (1986) and associated guidelines. All efforts were made to minimize any suffering and the number of animals used.

Surgical Procedure

Bilateral excitotoxic lesion of the ventral midline nuclei (NRE/Rh) (Experiment 1)

Before surgery all rats were anesthetized (isoflurane: induction 4%; maintenance 2-4%) and placed in a stereotaxic frame with the incisor bar set so as to achieve flat skull. The scalp was further anesthetized using lidocaine, cut and retracted. After craniotomy, excitotoxic lesions to the target region was made using N-methyl-D-aspartate (NMDA) dissolved in phosphate buffer, injected through a 1 µl Hamilton syringe at the following co-ordinates relative to

bregma: anterior-posterior (AP) -1.72mm and -2.40mm; mediolateral (ML) ±0.2mm; dorsoventral (DV) -7.40mm for both AP coordinates. NMDA (0.1µl and 0.09M) was injected gradually over 4 min and the needle left *in situ* for a further 4 min. For the sham surgeries, the animals underwent the same surgical procedures as the lesion group with the exception that no excitotoxin was injected once the needle had been lowered (n=10 for all groups).

Once surgery was completed the skin was sutured and an antibiotic powder (Acramide: Dales Pharmaceuticals, Skipton, UK) applied. All animals received at least 5 ml of glucose saline subcutaneously and systemic analgesia intramuscularly (0.05 ml Vetegesic: Reckitt Benckiser, Hull, UK) before the end of surgery. Hypromellose eye drops (Tubilux Pharma S.p.A, Pomazia, Italy) were given at the beginning and end of surgery. Animals recovered for at least 14 days before habituation to the behavioural arena commenced.

Cannulae implantation in the NRe (Experiments 2-5)

Following induction of anaesthesia as described above, rats were implanted with a single cannula aimed at NRe. The stainless steel guide cannula (26 gauge, Plastics One, Bilaney Consultants, UK) were implanted through a burr holes in the skull at the following co-ordinates relative to skull at bregma: AP -2.3mm, ML ±1.7mm; DV -6.2 mm (relative to surface of the skull) with the manipulator arm at an angle of 15° to the vertical (co-ordinates based on Cholvin et al 2013). The cannula was anchored to the skull by stainless steel skull screws (Plastics One, Bilaney Consultants, UK) and dental acrylic. Following surgery, each animal was given fluid replacement therapy and analgesia as described above and were housed individually for seven days to recover from surgery and were housed in pairs thereafter. The animals recovered for at least 14 days before habituation to the testing arena began.

Between infusions 33 gauge obdurators (Plastics One, Bilaney Consultants, UK) were used to keep the cannula patent.

Histology

On completion of the behavioural tasks animals were sacrificed by transcardial perfusion with phosphate buffer (PB) followed by 4% paraformaldehyde (PFA). The brains were post-fixed in 4% PFA for a minimum of 24 h followed by 48 h in 30% sucrose in PB. Coronal sections (40 µm) were cut on a cryostat and the sections mounted directly onto gelatine coated slides, stained using cresyl violet, and coverslipped using DPX mounting medium. Slides were then viewed under a light microscope and the extent of lesion or location of the cannula recorded.

To assess the extent of the Ne/Rh lesion representative sections along the anterior-posterior axis at the following co-ordinates relative to bregma were selected: -1.53mm, -2.00mm, -2.45mm, -2.85mm, -3.25mm. For each section the area of each ventral midline thalamic nuclei was measured (Image J) and the measured area across the sections were summed for each nuclei to generate a total area for each nuclei. Lesion size (% lesion) was calculated for each thalamic nucleus in each animal in the lesion group by comparison to the mean nuclei area in the sham group.

To assess the position of the cannula, sections along the anterior-posterior axis of each rat brain between -1.8mm and -2.5mm (relative to bregma) were selected and compared with those in the rat brain atlas (Swanson, 1998).

Behavioural Apparatus

Behavioural testing took place in a wooden open-topped arena (50 cm high, 90 cm wide and 100 cm length), with grey walls and external black curtains, to a height of 1.5 m, to restrict

distal cues. The floor which was covered in sawdust in all experiments (unless stated otherwise) was cleaned between animals. Exploration was monitored using an overhead camera and recorded onto DVD. The amount of object exploration was determined using in-house counting software on a computer within the room. Objects were constructed from Duplo® (Lego UK, Slough, UK) and varied in colour and size from 9x8x7cm to 25x15x10 cm, and were too heavy to displace. New objects were used for every experiment.

Behavioural procedures

Habituation:

After being handled for a week, the animals were habituated to the arena without stimuli for 10-15 min daily for four days prior to the commencement of the behavioural testing, the animals were also habituated to the infusion procedure.

Object-in-place task

This task comprised a sample and test phases separated by a delay, the length of which depended on the experiment. In the sample phase each subject was presented with four different objects (A, B, C, D). These objects were placed in the corners of the arena 10 cm from the walls (see Figure 1A). In this experiment one of the walls of the arena was black in colour, while the other three were grey and the curtains were partially removed from around the arena to provide additional extra maze cues. Each subject was placed in the centre of the arena and allowed to explore the objects for 5 min. During the delay period (which varied depending on the experiment or drug infused, see results for details) objects were cleaned with alcohol to remove olfactory cues and any sawdust which had stuck to the object. In the

test phase, two of the objects e.g. B and D (which were both on the left or right of the arena) exchanged positions (see Figure 1A) and the subject was allowed to explore the objects for 3 min. The time spent exploring the two objects that had changed position was compared to the time spent exploring the two objects that had remained in the same position. The objects moved (i.e. those on the left or right) and the position of the objects in the sample phase were counterbalanced between rats. If object-in-place memory is intact the subject will spend more time exploring the two objects which are in different locations, compared to the two objects which are in the same locations.

Object location task

Object location testing was conducted with the arena in the same configuration as for the object-in-place task (i.e. three grey coloured walls and one black wall, and the curtain around the maze was partially removed to provide the animals with access to extra maze cues). In the sample phase, animals were allowed to explore two identical copies of an object for 4 min before being removed from the arena for the 3 hr delay period. Following the delay, the animals were placed back into the arena which now contained two objects identical to those used in the sample phase (see Figure 1B). One object was replaced in the location previously occupied by a sample phase object, but the other object was placed in a new location within the arena. Object exploration was recorded for 3 min. The position of the moved object was counterbalanced across rats. If memory for the objects' original location is intact, animals will spend longer exploring the object in the novel location.

Novel object preference task

The novel object preference procedure comprised a sample and test phases separated by a delay. In the sample phase, duplicate copies of an object (e.g. A1 and A2) were placed near the two corners at either end of one side of the arena (see Figure 1C). The animal was placed into the arena facing the centre of the opposite wall and allowed a total of either 40 sec of exploration of A1 and A2, or 4 min in the arena. The retention delay varied depending upon the experiment (see results). At test (3 min duration), the animal was replaced in the arena, presented with two objects using the same positions as at acquisition: one object (A3) was the third copy of the object used in the sample phase and the other was a novel object (B3). The positions of the objects in the test and the objects used as novel or familiar were counterbalanced between the animals. If memory for the object encountered in the sample phase is intact animals will spend longer exploring the novel compared to the familiar object.

Drug Infusions

General procedures followed Barker et al. (2008). The drugs used were: muscimol, 2-amino-5-phosphonopentanoic acid (AP5, Tocris Bioscience, Bristol UK), scopolamine hydrobromide (Sigma-Aldrich), mecamylamine, anisomycin (Abcam, Cambridge, UK) and actinomycin D (Tocris Bioscience, Bristol, UK). Muscimol, AP5, scopolamine and mecamylamine were dissolved in sterile 0.9% saline solution, anisomycin was dissolved in equimolar hydrogen chloride and the pH was corrected to 7.4 with sodium hydroxide, actinomycin D was dissolved in DMSO at a concentration of 50mM and diluted using sterile 0.9% saline to the infusion concentration of 50µM, thus the concentration of DMSO infused was 0.1%. Vehicle infusions consisted of sterile 0.9% saline or saline plus 0.1% DMSO (actinomycin D). Muscimol was infused at a concentration of 2.4mM, AP5 at 25mM, scopolamine at 26mM, mecamylamine at 50µM, anisomycin at47mM, actinomycin D at 50µM.

Infusions were made through a 33 gauge cannula (Plastics One, Bilaney Consultants, UK) inserted into the implanted guide cannulae and extended 1mm beyond the end of the guide cannula and attached to a 5µl Hamilton syringe via polyethylene tubing. A volume of 0.3µl of fluid was injected into the NRe over a 1 min period. All intracerebral injections were made by infusion pump (Harvard Bioscience, Holliston, Mass.). To examine effects of manipulations on encoding, the infusions were made prior to the sample phase, to examine effects of manipulations on retrieval, infusions were made prior to the test phase. Following the infusion period, the infusion cannula remained in place for a further 5 min, before being removed. The animal was placed in the arena and the sample or test phase began 9 min later, i.e. 15 min after the start of the infusion. The time points for microinjection were based on reports that muscimol (at a higher concentration and volume than was used in the present study) takes effect within minutes and neuronal activity, with the exception of those neurons at the centre of the injection, will have recovered within 3-3.5 hr (Arikan et al., 2002; van Duuren et al., 2007)

Experimental design and statistical analysis

In Experiment 1 twenty animals were randomly assigned into the Sham (n=10) or NRe/Rh lesion groups (n=10). In Experiments 2-5 12 animals received bilateral infusions of drug or vehicle using a cross over design, and each animal was re-tested following a minimum rest period of 48 hr. Thus each animal served as its own control. Cannulae blockage resulted in the occasional loss of an animal (indicated by reduced degrees of freedom in the quoted statistical tests). Analysis of data from previous experiments in our laboratory indicate that a sample size of 8 will give a power of 0.8. Larger sample sizes were used to ensure statistical power was maintained if animals were lost from the analysis.

All measures of exploration were made with the experimenter blind to the lesion or drug status of each animal. Exploratory behaviour was defined as the animal directing its nose towards the object at a distance of < 2 cm. Any other behaviour such as looking around while sitting on or resting against the object was not considered as exploration. Discrimination between the objects was determined using a discrimination ratio (DR), calculated as the as the difference in time spent by each animal exploring the novel object, or objects in novel configuration, or object in novel location compared to the familiar object/ familiar configuration or familiar location, divided by the total time spent exploring all objects. This measure therefore takes into account individual differences in the total amount of exploration between rats (Ennaceur and Delacour, 1988; Dix and Aggleton, 1999). Comparisons of DR were made using a multi-factor analysis of variance (ANOVA) followed by simple main effects analysis. The variables, lesion (Experiment 1), delay (Experiment 1,2,3 and 5) and infusion timing (Experiment 4) were treated as between subjects factors drug whereas drug (Experiments 2-5) was treated as a with-in subjects factor. Additional analyses in both experiments examined whether individual groups had discriminated between the objects, using a one-sample t-test (two-tailed) comparing the DR against chance performance (DR= 0). All statistical analyses were performed using SPSS (IBM).

RESULTS

Histology

Experiment 1

One lesion and one sham animal died post-operatively, therefore n= 9 for each condition for all experiments. Histological analysis revealed that all remaining animals had substantial cell loss within the nucleus reuniens (NRe) and rhomboid nuclei (Rh). The largest and smallest of the lesions is shown in Figure 2A. The greatest tissue loss within the ventral midline nuclei was found in the NRe (mean ±sem 72%±5.6%, max 93%, min 41%), while the Rh and perireuniens nuclei sustained less damage (Rh: 33%±6.3%, max 64%, min 4%; perireuniens 28%±7.3, max 72%, min 0%). Comparison of the damage across the anterio-posterior extent of the lesion revealed significant cell loss in the anterior regions (see Figure 2A). Thus at 2.0mm posterior to bregma the mean cell loss was as follows NRe: 88% ±6.7%, Rh: 58%±10.2%, perireuniens nuclei: 43% ±11.8%. At 3.25mm posterior to bregma, the mean cell loss in NRe was 43% ±7.3%, Rh 10% ±4.7% and perireuniens 1.4% ±4.9%.

Outside of the nucleus reuniens and rhomboid nucleus there was limited cell loss in other thalamic nuclei. Two cases sustained damage in the submedial nucleus (SMT) (cell loss of 59% and 55%) while in all other cases the cell loss in SMT was more restricted (>30%). Additional cell loss occurred in the anteromedial nucleus, mediodorsal nucleus, paraventricular nucleus, central medial nucleus, zona incerta, ventral medial nucleus, paracentral nucleus, although in all cases the cell loss was less than 10% of the structure.

Experiments 2-5:

Histological examination confirmed that the tips of the cannulae were in the NRe as shown in Figure 2B

Experiment 1

Lesions in the NRe/Rh impair long-term associative recognition memory, but are without effect on single item recognition or spatial discrimination

The effect of lesions in the NRe/Rh on object-in-place memory was tested following a 5 min or 3 hr delay, and on object recognition and object location tasks following a 3 hr delay. The performance of the SHAM and NRe/Rh groups, are shown in Figure 3. Performance in the object-in-place task (Figure 3A) was significantly impaired in the NRe/Rh lesion group in a delay-dependent manner. ANOVA revealed a significant lesion x delay interaction ($F(1,33)=17.76$, $p=0.000$) and a significant main effect of delay ($F(1,33)= 19.71$, $p=0.000$). There was no significant main effect of lesion ($F(1,33)= 3.89$, $p=0.057$ n.s.). Analysis of the simple main effects confirmed that performance of the NRe/Rh group was significantly worse than the SHAM group at the 3 hr retention delay ($p=0.0005$). Comparison of the groups' performance against chance revealed that the SHAM group showed significant discrimination between the moved and unmoved objects at both delays [5 min ($t(8)= 4.69$; $p=0.002$; 3 hr delay $t(8)= 4.39$, $p=0.002$]. In contrast the NRe/Rh lesion group showed significant discrimination following the 5 min ($t(9)= 11.06$, $p=0.000$), but not the 3 hr retention delay ($t(8)= -0.93$, $p=0.380$ n.s.).

Performance of the SHAM and NRe/Rh groups in the object recognition and object location memory tasks was examined with a 3 hr delay between sample and test, as the NRe/Rh group showed impaired object-in-place performance following this delay. There was no difference between the two groups in object recognition performance (Figure 3B) main effect of lesion ($F(1,16)= 0.44$, $p=0.517$ n.s.) or in object location memory performance (Figure 3B main effect of lesion $F(1,16)= 0.06$, $p=0.805$ n.s.). Further both groups demonstrated significant discrimination between the novel and familiar in the object recognition (SHAM $t(8)= 9.51$,

p=0.000; NRe/Rh t(8)= 5.15, p=0.001) and object location tasks (SHAM t(8)= 5.06, p=0.001; NRe/Rh t(8)= 11.638, p=0.000).

Total object exploration levels in the sample and test phases of each of the tasks did not differ between the SHAM and NRe/Rh groups (see Table 1). Analysis of the sample phase exploration during the object-in-place task revealed a no significant lesion x delay interaction (F(1,33)= 0.03, p=0.876 n.s.) , main effect of lesion (F(1,33)= 1.22, p=0.278 n.s.) or delay (F(1,33)= 3.35, p=0.076 n.s.). Analysis of test phase exploration revealed no significant lesion x delay interaction (F(1,33)= 0.08, p=0.776 n.s.) and no significant main effect of lesion (F(1,33)= 1.16, p=0.289 n.s.), however there was a significant main effect of delay (F(1,33)= 4.48, p=0.042) accounted for by an increase in object exploration in both conditions at the 3 hr delay.

ANOVA of the total object exploration completed in either the object recognition and object location tasks revealed no significant differences in either the sample phase (object recognition F(1,16)= 0.29, p=0.866 n.s.; object location F(1,16)= 1.90, p=0.187 n.s.) or test phase (object recognition F(1,16)= 0.53, p=0.478 n.s.; object location F(1,16)= 0.58, p=0.458 n.s.).

Thus, these data suggest that the midline thalamic nuclei which include the nucleus reuniens are critical for object-in-place associative recognition memory performance following a 3 hr, but not a 5 min retention delay.

Experiment 2

Muscimol inactivation of the NRe impaired both encoding and retrieval of long-term associative recognition memory

We next examined whether temporary inactivation of the NRe disrupted either encoding or retrieval of object-in-place recognition memory. Infusions (1 min duration) occurred either 15 min before the sample phase, so as to inactivate the NRe during encoding, or 15 min before the test phase so as to inactivate the NRe during retrieval. The effect of muscimol on encoding object-in-place memory was tested following a 5 min or 3 hr delay, the effect of muscimol on retrieval was tested following a 3 hr delay only.

Pre-sample phase infusion of muscimol into the NRe produced a delay-dependent deficit in object-in-place memory (Figure 4) confirmed by a significant drug x delay interaction $F(1,22)= 6.02$, $p=0.023$ and main effect of delay ($F(1,22)= 7.87$, $p=0.010$), but no significant main effect of drug ($F(1,22)= 4.15$, $p=0.054$ n.s.). Analysis of the simple main effects revealed a significant difference between the muscimol and vehicle infused animals at the 3 hr delay only ($p=0.000$). Comparison of the groups' discrimination performance against chance revealed that the vehicle and muscimol animals significantly discriminated between the moved and unmoved objects at the 5 min delay [vehicle ($t(11)=5.62$, $p=0.000$) muscimol ($t(11)= 5.50$, $p=0.000$)] but not at the 3 hr delay, [vehicle ($t(11)= 6.12$, $p=0.000$) muscimol ($t(11)= 0.79$, $p=0.446$ n.s.)].

Pre-test infusion of muscimol significantly impaired object-in-place performance (Figure 4; main effect of drug $F(1,11)= 51.42$, $p=0.000$) confirmed by comparison of each group's performance against chance which revealed that vehicle-infused ($t(11)= 7.10$, $p=0.000$) but not muscimol-infused animals ($t(11)= -0.90$, $p=0.389$ n.s.) showed significant discrimination.

Analysis of the total object exploration completed in the sample and test phases of the object-in-place task revealed no significant differences between the muscimol and vehicle treated groups (see Table 2 for means). ANOVA of the total object exploration in the test phase following pre-test phase infusions revealed no significant drug x delay interaction ($F(1,22)= 2.73$, $p=0.113$ n.s.) or main effect of drug ($F(1,22)= 0.20$, $p=0.663$ n.s.), but there was a significant main effect of delay ($F(1,22)= 14.70$, $p=0.001$) as there was an increase in total object exploration following the 3 hr delay in both groups.

Thus, both the encoding and retrieval of long-term object-in-place associative recognition memory is dependent on neuronal activation with the NRe.

Experiment 3

NMDA receptor transmission in the NRe is not required for long-term associative recognition memory

To examine whether object-in-place memory encoding is dependent on NMDAR neurotransmission in the NRe, the NMDA receptor antagonist AP5 was infused into the NRe prior to the sample phase and memory performance was assessed following a 3 hr or 24 hr delay.

Infusion of AP5 into the NRe before the sample phase had no effect on object-in-place performance at either retention delay (Figure 5). Two-way ANOVA with drug and delay as factors found no significant interaction ($F(1,22)= 0.001$, $p=0.984$ n.s.) and no significant main effect of drug ($F(1,22)= 1.27$, $p=0.273$ n.s.) or delay ($F(1,22)= 0.17$, $p=0.688$ n.s.). Further analysis confirmed that both groups significantly discriminated following the 3 hr (vehicle

t(11)= 6.64, p=0.001; AP5 t(11)= 6.53, p=0.001) and 24 h (vehicle t(11)= 6.49, p=0.001; AP5 t(11)= 5.31, p=0.001) retention delays.

AP5 did not significantly alter total object exploration levels in either the sample or test phase (see Table 2 for means). Analysis of sample phase exploration revealed no significant drug x delay interaction($F(1,22)= 0.42$, $p=0.524$ n.s.) and no significant main effect of drug ($F(1,22)= 0.25$, $p=0.620$ n.s.) or delay ($F(1,22)= 0.70$, $p=0.410$ n.s.). Analysis of the total test phase object exploration revealed no significant drug x delay interaction ($F(1,22)= 0.18$, $p=0.679$ n.s.) and no significant main effect of drug ($F(1,22)= 0.01$, $p=0.911$ n.s.) or delay ($F(1,22)= 2.93$, $p=0.101$ n.s.).

These results show that NMDA receptor activation in the NRe is not required for associative recognition memory encoding.

Experiment 4

Cholinergic receptor transmission in the NRe is required for encoding but not retrieval of associative recognition memory

The NRe contains a dense population of both nicotinic (Clarke et al., 1985) and muscarinic cholinergic receptors (Frey et al., 1985; Frey and Howland, 1992), so to assess the importance of cholinergic neurotransmission in the NRe for object-in-place memory, the non-competitive muscarinic receptor antagonist scopolamine or the nicotinic antagonist mecamylamine were infused prior to the sample or test phase. Memory performance was assessed following a 3 hr delay .

Scopolamine impaired object-in-place performance when infused prior to the sample but not prior to the test phase (Figure 6A). Two-way ANOVA with drug and infusion timing as factors

revealed a significant interaction ($F(1,22)= 10.50$, $p=0.004$) and a significant main effect of drug ($F(1,22)= 10.87$, $p=0.003$) and infusion timing ($F(1,22)= 7.455$, $p=0.012$). Analysis of the simple main effects revealed a significant difference between the performance of the vehicle and scopolamine infused animals when infusions occurred before the sample phase only ($p<0.001$). Further analysis confirmed that the vehicle infused animals significantly discriminated between the moved and unmoved objects at both infusion time points (pre-sample $t(11)= 5.04$, $p=0.000$; pre-test $t(11)= 7.39$, $p=0.000$), in contrast scopolamine-infused animals did not discriminate when the infusion occurred before the sample phase ($t(11)= -0.26$, $p=0.797$ n.s.), but showed significant discrimination when the infusion occurred before the test phase ($t(11)= 5.02$, $p=0.000$).

Infusion of mecamylamine impaired object-in-place performance when infused prior to the sample, but not prior to the test phase (Figure 6B). Two-way ANOVA revealed a significant drug x infusion timing interaction ($F(1,22)= 21.15$, $p=0.000$) and significant main effects of drug ($F(1,22)= 20.36$, $p=0.000$) and infusion timing ($F(1,22)= 13.13$, $p=0.002$). Analysis of the simple main effects revealed a significant difference between the performance of the vehicle and mecamylamine infused animals when infusion occurred before the sample phase only ($p<0.001$). Further analysis confirmed that the vehicle-infused animals showed significant discrimination between the moved and the unmoved objects under both infusion conditions (pre-sample $t(11)= 5.30$, $p=0.000$; pre-test $t(11)= 7.19$, $p=0.000$), in contrast mecamylamine disrupted discrimination when administered pre-sample ($t(11)= -1.71$, $p= 0.116$ n.s.) but not when administered pre-test ($t(11)= 7.64$, $p=0.000$).

Overall object exploration levels were not significantly altered by either scopolamine or mecamylamine (Table 3). Analysis of the total amount of object exploration in the sample

phase revealed a non-significant drug x infusion timing interaction following scopolamine ($F(1,22)= 1.07$, $p=0.312$ n.s.) or mecamylamine administration ($F(1,22)= 0.19$, $p=0.664$ n.s.). Analysis of the total amount of object exploration completed in the test phase again revealed a non-significant drug x infusion timing interaction following scopolamine ($F(1,22)= 3.03$, $p=0.096$ n.s.) or mecamylamine ($F(1,22)=0.32$, $p=0.576$ n.s.).

These results show that activation of both muscarinic and nicotinic cholinergic receptors in the NRe is essential for encoding but not retrieval of associative recognition memory.

Experiment 5

Associative recognition memory depends on protein synthesis in the NRe

Given that the experiments revealed a time-dependent role for the NRe in object-in-place memory we next examined whether such long-term memory maintenance was dependent on protein synthesis. The protein synthesis inhibitor anisomycin was infused into the NRe before the sample phase and object-in-place recognition was tested following a 3 hr and 24 hr delay. Memory performance at the two retention delays is shown in Figure 7A. Two-way ANOVA with drug and delay as factors revealed a significant interaction ($F(1,20)=5.07$, $p=0.036$) and significant main effects of both treatment ($F(1,20)= 13.42$, $p=0.002$) and delay ($F(1,20)= 7.10$, $p=0.015$). Analysis of the simple main effects revealed that anisomycin treatment significantly impaired memory performance following the 24 hr delay ($p<0.001$) but not the 3 hr delay, and performance of the anisomycin-infused animals was significantly different at the two delays ($p<0.01$). Further analysis confirmed that the vehicle infused animals showed discriminated between the moved and unmoved objects at both delays tested (3 hr $t(10)= 4.50$, $p=0.001$; 24 h $t(10)= 5.90$, $p=0.000$), in contrast anisomycin infused

animals did show significant discrimination at the 3 hr delay ($t(10)= 2.99$, $p=0.014$) but not at the 24 hr delay ($t(10)= -1.02$, $p=0.330$ n.s.).

Anisomycin is reported to cause apoptosis (Iordanov et al., 1997;1998), so the experiment was repeated using the protein synthesis inhibitor actinomycin D. Analysis of the DR (Figure 7B) revealed that actinomycin D significantly impaired object-in-place performance (mean DR vehicle= 0.49±0.08, actinomycin=-0.07±0.08) confirmed by a significant main effect of drug ($F(1,9)= 22.13$, $p=0.001$). The vehicle-infused animals discriminated between the moved and unmoved objects ($t(9)=5.96$, $p=0.000$) while drug treated animals did not ($t(9)=-0.82$, $p=0.435$ n.s.).

Analysis of the total object exploration (Table 4) revealed that neither protein synthesis inhibitor infused prior to the sample phase had any effect on exploration [anisomycin drug x delay interaction $F(1,20)=0.17$, $p=0.681$ n.s.; main effect of drug ($F(1,20)= 1.61$, $p=0.219$ n.s.; main effect of delay ($F(1,20)= 0.09$, $p=0.767$ ns; actinomycin D ($F(1,9)= 0.13$, $p=0.727$ n.s.)].

Analysis of the total object exploration completed in the test phase following anisomycin revealed no effect of drug ($F(1,20)= 0.47$, $p=0.500$ n.s.), delay $F(1,20)= 1.03$, $p=0.323$ n.s.) or drug x delay interaction ($F(1,20)= 4.27$, $p=0.052$ n.s.). Actinomycin D also had no effect on exploration during the test phase ($F(1,9)= 4.17$, $p=0.072$ n.s.).

These results show that long-term associative recognition memory is dependent on protein synthesis in the NRe.

DISCUSSION

Permanent lesions in the ventral midline nuclei, i.e NRe/Rh, produced a delay dependent impairment in object-in-place memory, but had no effect on novel object recognition or object location memory. Muscimol infusion specifically into NRe also had no effect on short-term memory but when the retention delay was 3 hr, infusions prior to the sample or prior to the test phase impaired performance indicating that both encoding and retrieval phases of object-in-place memory are dependent on the NRe. Infusion of AP5 during the sample phase had no effect on memory performance, in contrast both scopolamine and mecamylamine administered prior to the sample phase impaired performance following a 3 hr delay, thus long-term object-in-place memory does not require NMDA receptor transmission but is dependent on cholinergic receptor neurotransmission through both muscarinic and nicotinic receptors. Finally, inhibition of protein synthesis impaired memory performance following a 24 hr but not 3 hr delay, suggesting that the NRe participates in long-term object-in-place memory consolidation.

The NRe in an associative memory circuit

The NRe plays a selective role in long-term object-in-place associative memory as shown by performance deficits following both permanent and temporary inactivation. The latter finding not only confirms the delay-dependent role played by the NRe, but also allows us to exclude the possibility that intact short-term memory was due to the development of compensatory processes, such as might occur through neural re-organisation between surgery and behavioural testing.

Associative recognition is dependent on a neural circuit involving the HPC, mPFC and PRH (Barker et al., 2007; Barker and Warburton, 2011) to which the NRe has bi-directional

connections (Vertes et al., 2006). Patterns of neuronal activation in the NRe have been shown to have a direct influence on neurons in the HPC (Dolleman-van-der Weel et al., 1997; 2016) to which it provides an excitatory glutamatergic input (Dolleman-van der Weel and Witter, 2000; Van der Werf et al., 2002; Vertes et al., 2006; Pereira et al., 2016), however lesions in the NRe had no effect on the hippocampal-dependent object location memory task (Barker and Warburton, 2011). NRe lesions also had no effect on the perirhinal-dependent single item recognition memory task, thus, the object-in-place deficits are unlikely to reflect a loss of NRe input solely to either the HPC or PRH. The NRe provides a glutamatergic input to the mPFC (Eleore et al., 2011; Di Prisco and Vertes, 2006) but although mPFC lesions impair object-in-place memory (Barker et al., 2007) other studies have suggested that NRe activity is not important for tasks that depend solely on the mPFC, such as a visual serial reaction time task (Hembrook and Mair, 2011). There are, however, an increasing number of studies that show that the NRe has a critical role in tasks the cognitive demands of which require *both* HPC and mPFC possibly because the NRe provides a route of communication between these regions in the absence of a direct mPFC-HPC pathway, or because the NRe can modulate activity in both regions simultaneously (Hoover and Vertes, 2012; Hallock et al., 2016). The impairments in object-in-place memory observed in the present study may be explained by either of these suggestions, although as HPC-mPFC communication is required for object-in-place performance at both short- and long-term retention delays (Barker and Warburton, 2008) but here memory impairments were only seen at the 3h delay, it is unlikely that the NRe acts as a simple relay station between the HPC and mPFC. While previous studies have also reported a delay-dependent deficits following NRe lesions (Loureiro et al., 2012), arguing that this effect is a result of an increased requirement for HPC-mPFC cross talk at longer delays, these tasks examined spatial working memory and thus employed considerably

shorter delays than those used here. Thus, the available evidence points to an important role for the NRe in co-ordinating activity between the mPFC and HPC (Hallock et al., 2016), but the mPFC and HPC also each interact with the PRH during associative recognition memory formation. Given the reciprocal connections between the NRe and PRH (Agster et al., 2016; Pereira et al 2016), the NRe could be critical for co-ordinating activity between all three brain regions and at present there is no direct evidence to either support or refute this possibility.

Infusion of muscimol prior to either the sample phase or test phase impaired long-term object-in-place showing that the NRe supports both encoding and retrieval. Reports of the effects of muscimol, albeit at higher volumes and concentrations than used in the present study, suggest that neuronal suppression can last several hours following an infusion (van Duuren et al., 2007; Arikan et al., 2002). Thus, it is possible that, following the pre-sample infusion of muscimol in the present study, some NRe tissue remains inactive during the test phase. Evidence suggests however, that following a 3 hr delay only a small proportion of neurons close to the infusion site will still be affected (Arikan et al., (2002) and so are unlikely to account for the significant deficits observed.

Electrophysiological recording studies have shown that encoding of object-place associative information requires the flow of information from the HPC to the mPFC, (Place et al., 2016) while retrieval requires mPFC to HPC information trnasfer (Place et al., 2016) via the NRe (Hallock et al., 2016;). The NRe maintains the specificity of memory information processing through modulation of both HPC and mPFC activity (Dolleman-van der Weel et al., 2009; Ito

et al., 2015) but in these studies the pattern of NRe activity during the behavioural tasks was found to be independent of memory requirements, suggesting that the NRe itself does not process memory information. While our observation that NMDA receptor blockade in the NRe had no effect on memory, while such blockade in the HPC or mPFC produced significant impairments (Barker and Warburton, 2008) is somewhat consistent with the view that the NRe does not process mnemonic information, our results that the maintenance of object-in-place associative memory, was dependent on protein synthesis, does suggest that the NRe is involved in object-in-place memory consolidation and stablization (Pereira de Vasconcelos and Cassel, 2015). Whether the synthesis of new proteins occurs in the cortical- or hippocampal-projecting NRe cells or in a third cell population remains to be established.

Cholinergic receptor mediated neurotransmission in the NRe was found to be critical for object-in-place memory but while this region contains both nicotinic (Clarke et al., 1985) and muscarinic receptors (Wamsley et al., 1984; Cortes and Palacios, 1986; Mash and Potter, 1986) their precise distribution has yet to be described. The cholinergic input to the NRe arises from the pedunculopontine (PPT) and laterodorsal tegmental nuclei (Woolf and Butcher, 1986; Cornwall et al., 1990), thus its cholinergic innervation is clearly distinct from that of the HPC or PFC which arises in the basal forebrain. Salient environmental stimuli increase PPT cholinergic neuron firing, which in turn results in a depolarization of thalamocortical neurons (McCormick and Bal, 1997; Steriade et al, 1997; 2004) via both nicotinic and muscarinic receptors. Hence cholinergic input to the thalamic nuclei may provide an important alternative route by which hippocampal or cortical function may be modulated during memory formation, further experiments are clearly necessary to establish how precisely this is achieved.

It has been argued that, through its projections to the mPFC and HPC, the NRe can regulate attentional vigilance and cortical arousal (Van der Werf et al., 2002; Vertes et al., 2007; Steriade *et al*., 1990, Steriade *et al*., 1997). In the present study, no changes in general exploratory activity were found, and the memory impairments were specific, i.e. there were no deficits in the novel object recognition or object location tasks. Hence it is unlikely that the object-in-place memory impairments, following NRe inactivation, or blockade of cholinergic neurotransmission are explained by non-specific changes in arousal or attention. Rather the results indicate a role for NRe in regulating HPC-mPFC interactions under conditions of greater mnemonic demand, possibly by stabilising memory traces within the network.

The NRe can now be incorporated as a key node within a recognition memory network that includes the HPC, mPFC and PRH, although its role is selectively in long-term memory formation and retrieval. These results together with the existing literature suggest the NRe coordinates activity across HPC and mPFC, although the mechanism by which this is achieved may depend on the stages of memory processing. Indeed, the NRe is likely to play a diverse role and the delay-dependence of its involvement argues strongly against a model in which it is a simple relay between the subcortical and cortical structures. Rather the data suggest that the NRe is an important facilitator of HPC-mPFC interactions and that within the NRe encoding of mnemonic information is mediated by cholinergic neurotransmission. Long-term associative memory stability within the network is clearly mediated by a protein synthesis dependent mechanism. While the NRe must be included within the associative recognition memory circuit, it is now necessary to conduct detailed investigations of the direction of

information transfer between the mPFC, HPC and NRe to establish the mechanism by which NRe provides important regulation of long-term memory processing.

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